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Citation for published version:

Borges, LE, Horne, AW, McDonald, SE, Shaw, JLV, Lourenco, PC, Petraglia, F & Critchley, HOD 2011, 'Attenuated tubal and endometrial urocortin 1 and corticotropin-releasing hormone receptor expression in ectopic pregnancy', *Reproductive Sciences*, vol. 18, no. 3, pp. 261-8.
<https://doi.org/10.1177/1933719110385132>

Digital Object Identifier (DOI):

[10.1177/1933719110385132](https://doi.org/10.1177/1933719110385132)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Reproductive Sciences

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Published in final edited form as:

Reprod Sci. 2011 March ; 18(3): 261–268. doi:10.1177/1933719110385132.

Attenuated tubal and endometrial urocortin 1 and corticotrophin releasing hormone receptor expression in ectopic pregnancy

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Abstract

Fallopian tube (FT) and endometrial urocortin 1 (Ucn1) and CRH-receptor (CRH-R1/CRH-R2) expression were examined using quantitative-RT-PCR and immunohistochemistry in non-pregnant and pregnant women (intrauterine, IUP; ectopic pregnancy, EP). Tubal Ucn1 mRNA expression was higher in luteal compared to follicular phase ($P < 0.01$) and equivalent to follicular phase in FT from EP. Tubal CRH-R1/CRH-R2 mRNA was lower in luteal phase ($P < 0.05$) and in FT from EP compared to follicular phase ($P < 0.01$). Ucn1 mRNA was lower in endometrium from EP compared to IUP ($P < 0.05$). CRH-R1 mRNA was higher in endometrium from EP compared to viable IUP ($P < 0.05$). No differences were observed in CRH-R2 expression. CRH-R1 protein was primarily localised to epithelium of FT and endometrium. Quantitative analysis of tubal CRH-R1 protein expression reflected that seen at the mRNA level but endometrial expression was equivocal. The demonstration of attenuated tubal/endometrial Ucn1/CRH-R expression in EP further supports a role of the CRH-family in embryo implantation.

Keywords

Urocortin 1; corticotrophin releasing hormone receptor; Fallopian tube; endometrium; ectopic pregnancy

Introduction

Tubal ectopic pregnancy remains a common cause of morbidity and occasional mortality [1]. In the UK, between 2003 and 2005, early pregnancy bleeding was the third commonest cause of maternal death and over 60% of these cases were due to ruptured tubal ectopic pregnancies [2]. In the USA, ruptured tubal ectopic pregnancy remains the commonest cause of pregnancy-related first trimester death [3]. In the developing world, the incidence of ectopic pregnancy is much higher and one in ten women admitted with a diagnosis of ectopic pregnancy ultimately die from the condition [4]. Despite advances in our

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Conflict of Interest:

Authors declare they have no competing financial interests.

understanding of intrauterine implantation and early pregnancy, our knowledge of the complex molecular and cellular interactions that contribute to tubal implantation is limited [5].

The corticotrophin releasing hormone (CRH) family includes a variety of neuropeptides, including urocortin 1 (Ucn1), and the CRH receptors (types 1 and 2) (CRH-R1 and CRH-R2) [6]. Ucn1, a 40 amino acid peptide, is closely related to CRH [7], not only in sequence homology but also because it binds both CRH receptors with high affinity [8,9]. Ucn1 is known to play a significant role in the physiology of human reproduction, including steroidogenesis, myometrial contractility and the initiation of labour [6,10,11]. Ucn1 and the CRH receptors are also thought to play an important part in endometrial decidualization and successful intrauterine implantation [6,10]. Ucn1 is expressed in the endometrial epithelial and stromal cells throughout the menstrual cycle [12], with the highest expression observed in the secretory phase [13]. It induces endometrial cell decidualization even in the absence of sex steroid hormones, and is also secreted by the stroma during decidualization modulating the maternal response to the invading trophoblast [13].

Ucn1 is highly expressed by human extravillous trophoblast cells (EVT) (the invasive part of the placenta) [14] and it has been reported to have a potent vasodilator action on the placental bed [15]. Furthermore, higher Ucn1 levels have been observed in endometrial flushings from women who achieved pregnancy after intrauterine insemination compared to those who did not, suggesting that Ucn1 may only be secreted in appreciable amounts by a receptive endometrium [16]. Both CRH-R1 and CRH-R2 [17,18] are also expressed in the human endometrium throughout the menstrual cycle but only CRH-R1 is expressed on EVT cells [19]. CRH-R2 is extensively expressed in vascular tissues [20,21], and its action on vascular tone control promotes vasodilation [22,23]. Recent studies have shown that, in the human endometrium, CRH-R1 antagonists have the potential to be used as abortive agents (blockage of CRH-R1 in rats decreases implantation in rats in a dose-dependent way) [19,24]. It has been also suggested that CRH-R1 takes part in the control of trophoblast invasion and embryo-maternal immuno tolerance by killing activated T cells expressing the Fas membrane protein at the fetal-maternal interface, an effect completely reversed by antalarmin, a specific CRH-R1 antagonist [19].

We believe that ectopic implantation in the Fallopian tube is manifest by an alteration in Ucn1 and CRH receptor expression reflecting pathological embryo invasion. We hypothesised that Ucn1 and CRH receptor mRNA expression in the non-pregnant Fallopian tube would be similar to that observed in the endometrium but different at tubal implantation sites and in the decidualized endometrium of women with tubal ectopic compared to intrauterine pregnancies.

Material and Methods

Tissue collection

Ethical approval for this study was obtained from the Lothian Research Ethics Committee (LREC 04/S1103/20), and informed written consent was obtained from all study participants. Timed Fallopian tube biopsies (all full-thickness from ampullary region) were obtained from health fertile women (age 19-40 years) with regular menstrual cycles (21-35 days) undergoing surgery for benign gynaecological conditions (e.g. heavy menstrual bleeding, adenomyosis) (n=12) (Table 1). Dating of the samples was ascertained by consistency of circulating serum estradiol and progesterone levels and histological analysis of an endometrial biopsy at time of sample collection (using Noyes' criteria). Fallopian tube was also collected from women undergoing surgery for tubal ectopic pregnancy (n=6). Decidualized endometrium was obtained from women (age 19-40 years) undergoing

surgical termination of pregnancy (viable intrauterine) (n = 10, mean gestation 67.4 d); surgical management of embryonic missed miscarriage (non-viable intrauterine) (n = 7, mean gestation 71.3 d), and surgical management of tubal ectopic pregnancy (n = 12, mean gestation 58.1 d). None of the pregnant women had undergone in-vitro fertilization and none of the women undergoing surgical management of tubal ectopic pregnancy presented acutely with hemodynamic shock, and all required serial serum β -hCG and ultrasound monitoring before diagnosis. None of the subjects had received medication (e.g. hormones or prostaglandins) in the five days leading up to the procedure or had an intra-uterine contraceptive device in-situ. The decidualized endometrium and trophoblast were obtained by suction curettage from the women with intrauterine pregnancies and by Pipelle™ (Eurosurgical Ltd., Cranleigh, UK) from the women with tubal ectopic pregnancies. The decidualized endometrium was isolated from the trophoblast macroscopically. Haematoxylin and eosin staining and immunohistochemistry for cytokeratin demonstrated clear isolation of the decidualized endometrium from the trophoblast as described previously [25]. Samples were either immersed in RNA later (Ambion, Inc., Austin, TX) at 4 °C overnight and flash frozen at -80 °C for RNA extraction, or immersed in 4% neutral buffered formalin for 24 hours then transferred into 70% ethanol and paraffin embedded for immunohistochemistry.

RNA extraction

Total RNA was extracted from all samples as detailed in the manufacturers' protocol (Qiagen, West Sussex, UK). The concentration and quality of the extracted RNA was assessed using an Agilent bioanalyser and quantified using an ND-1000 spectrophotometer (Nanodrop technologies, DE, USA). All samples were standardized for quality control and assigned an RNA integrity number (RIN). RNA samples were considered to be of good quality when a mean RIN value of 7.5 was obtained [26].

Quantitative RT-PCR

After DNase treatment, using RQ1 DNase (Promega, Southampton, UK), the RNA was reverse transcribed into cDNA using random hexamers (Applied Biosystems, Foster City, CA). TaqMan quantitative RT-PCR was then performed using Applied Biosystems' inventoried TaqMan Gene Expression Assay for the following genes: human urocortin 1 (Ucn1) (Hs00175020_m1), human CRH receptor type 1 (CRH-R1)(Hs01062290_m1) and human CRH receptor type 2 (CRH-R2) (Hs00266401_m1). All sample reactions were performed in triplicate and a relative comparison was made to a control tissue cDNA which was included in all reactions. Using the $2\Delta\Delta C_t$ method, Ucn1, CRH-R1 and CRH-R2 mRNA expression results were normalized against ribosomal 18S internal control (Applied Biosystems) and expressed as the fold-change when compared with the control group.

Serum assays

Serum progesterone and hCG concentrations were measured using a standard radioimmunoassay [27]. Levels were correlated to tissue mRNA levels of each gene using Excel.

Immunohistochemistry

Immunohistochemical localisation of CRH-R1 was performed using a goat polyclonal antibody (Santa Cruz Biotechnology), biotinylated secondary antibody and peroxidase conjugated detection. Immunoreactivity was visualised using the chromagen 3,3'-diaminobenzidine (DAB). Tissue sections were dewaxed in xylene and rehydrated in descending grades of alcohol. Sections were subjected to antigen retrieval in 0.01M sodium citrate and non-specific activity was blocked sequentially with 3% hydrogen peroxide (Sigma-Aldrich), avidin, biotin and protein blocks. Sections were incubated overnight at 4 °C

with primary antibody. Sections were subsequently incubated with biotinylated secondary antibody (Vector Laboratories, Peterborough, UK) and HRP complex (ABC-Elite, Vector Laboratories) then immunoreactivity detected using DAB (Vector Laboratories). Counterstaining was then performed with Harris' haematoxylin and mounted in Pertex (Cellpath Technologies, Hemel Hempstead, UK). Negative controls were included where the primary antibody was pre-incubated with a blocking peptide (Santa Cruz Biotechnology) and third trimester placenta was used as a positive control.

Scoring

For assessment of CRH-R1 immunostaining in Fallopian tube and endometrium, a histoscore was applied by two independent observers as previously described [28]. A separate histoscore was applied to surface epithelium and stroma where appropriate.

Statistical analysis

All data were assessed for normality of distribution using a computer programme (Prism 4; Graphpad Software, CA, USA). Where the data were normally distributed, and three or more groups were being analysed, an ANOVA with Tukey's Multiple Comparison Test was used. Where only two groups were being analysed, a t-test was used. A Chi-squared test was used to compare proportions. Spearman (non-parametric) coefficients were used to assess linear correlations. Histoscore was analysed using the Kruskal-Wallis test assuming a non-Gaussian distribution. $P < 0.05$ was taken to indicate statistical significance.

Results

Urocortin 1 (Ucn1) mRNA expression in non-pregnant human Fallopian tube and in Fallopian tube from women with ectopic pregnancy

In the non-pregnant human Fallopian tube, Ucn1 mRNA expression was higher in the progesterone-dominant luteal phase (mean ΔCT $14.76 \pm SEM$ 0.35 , $n=8$) compared to the follicular phase (16.44 ± 0.25 , $n=4$) ($p < 0.01$) (Figure 1). In Fallopian tube from women with ectopic pregnancy (where tubal implantation had occurred), Ucn1 mRNA expression was similar to that observed in the follicular phase (16.70 ± 0.43 , $n=6$) (Figure 1).

CRH receptor (types 1 and 2) mRNA expression in non-pregnant human Fallopian tube and in Fallopian tube from women with ectopic pregnancy

Both CRH receptors (types 1 and 2) assumed the same pattern of expression in the non-pregnant Fallopian tube and in Fallopian tube from women with ectopic pregnancy. Expression was higher in the follicular (CRH-R1 17.14 ± 0.28 ; CRH-R2 17.24 ± 0.36 ; $n=4$) compared to the luteal phase (CRH-R1 18.47 ± 0.29 ; CRH-R2 18.81 ± 0.43 ; $n=8$) ($p < 0.05$), and equivalent to (CRH-R1), or lower than (CRH-R2), the luteal phase in Fallopian tube from women with ectopic pregnancy (where tubal implantation had occurred) (CRH-R1 19.08 ± 0.23 ; CRH-R2 19.90 ± 0.29 ; $n=6$) ($p < 0.01$) (Figure 2).

Urocortin 1 (Ucn1) mRNA expression in decidualized endometrium from women with intrauterine and ectopic pregnancy

Ucn1 mRNA expression was lower in the decidualized endometrium from women with ectopic (18.48 ± 0.19 , $n=12$) compared to viable intrauterine pregnancy (16.63 ± 0.16 , $n=10$) ($p < 0.001$). No difference in decidual Ucn1 mRNA expression was observed between non-viable compared to viable intrauterine and ectopic pregnancy (Figure 3). The findings could not be explained by differences in gestational age.

CRH receptor (types 1 and 2) mRNA expression in decidualized endometrium from women with intrauterine and ectopic pregnancy

CRH-R1 mRNA expression was significantly higher in decidualized endometrium from ectopic (17.36 ± 0.87 , $n=12$) compared to viable intrauterine pregnancy (20.84 ± 0.43 , $n=10$) ($p<0.05$). No significant difference in expression was observed when compared to decidualized endometrium from non-viable intrauterine pregnancy (Figure 4). CRH receptor type 2 mRNA expression was not significantly different in the decidualized endometrium from the three groups analysed, but there was a non-significant trend for lower expression in the non-viable pregnancy group (Figure 4). The findings were correlated with gestation (days) but could not be explained by differences in gestational age within the study population.

CRH-R1 protein expression in Fallopian tube and decidualized endometrium

CRH-R1 protein was localised to the epithelium of the Fallopian tube (Figure 5a-c). Expression was cytoplasmic with nuclear sparing. There was no difference in localization at different times of the menstrual cycle or in Fallopian tube from women with ectopic pregnancy. CRH-R1 showed a similar pattern of expression in the decidualized endometrium although there was evidence of patchy cytoplasmic stromal expression (Figure 5d-f). There was no difference in localisation in the ectopic pregnancy compared to the intrauterine groups. CRH-R1 protein expression was quantified by applying a histoscore (0–300) (Figure 6a-c). None of the differences between each group were statistically significant. However, tubal epithelial CRH-R1 expression showed a non-significant trend reflecting CRH-R1 expression at the mRNA level. Expression was higher in the follicular compared to the luteal phase and lower than the luteal phase in Fallopian tube from women with ectopic pregnancy (where tubal implantation had occurred).

Correlation of decidual urocortin and CRH-R1 mRNA expression with serum progesterone and hCG concentrations

hCG and progesterone levels were measured in all patients with a viable intrauterine and ectopic pregnancy. The range of serum hCG concentrations was 22.772–185.815 IU/L for the women with the viable intrauterine pregnancies and 225–15.956 IU/L for the women with the ectopic pregnancies. The range of serum progesterone concentrations was 40.99–82.55 nmol/L for the women with the viable intrauterine pregnancies and 20.36–158.13 nmol/L for the women with the ectopic pregnancies. No clear correlation between decidual Ucn1 or CRH-R1 mRNA expression and serum hCG or progesterone was observed.

Discussion

To our knowledge, this is the first description of Ucn1 and CRH receptor mRNA expression in the non-pregnant human Fallopian tube at different stages of the menstrual cycle and in women with ectopic pregnancy. Furthermore, we also compare the expression of Ucn1 and CRH receptor mRNA in the decidualized endometrium of women with intrauterine compared to ectopic pregnancies.

Similar to human endometrium [13], we demonstrate that tubal Ucn1 mRNA expression is higher in the progesterone-dominant luteal compared to both the follicular phase. We also show that tubal expression in the luteal phase is higher than that observed in Fallopian tube from women with ectopic pregnancy where tubal implantation has occurred. The latter data may be due to the fact that tubal decidualization is not generally observed in ectopic pregnancy [29,30]. Ucn1 is highly expressed in the secretory endometrium [13] and in EVT

cells [14], promotes decidualization in-vitro [13], and is only produced in large amounts by an endometrium receptive to a presenting embryo [16].

In addition, we show that CRH-R1 and CRH-R2 mRNAs are expressed in non-pregnant Fallopian tube with statistically lower expression in the luteal compared to the follicular phase. Tubal CRH-R1 protein expression showed a similar non-statistically significant trend. In the endometrium, the CRH receptors are expressed throughout the menstrual cycle but cyclical variations have not been reported [18]. Since CRH-R1 promotes implantation through an inflammatory-like reaction of the endometrium [19], and CRH-R2 promotes vasodilation within the placental bed [22,23], the reduced expression observed in the Fallopian tube during the luteal phase may in theory act as a barrier to unwanted ectopic implantation.

Furthermore, we demonstrate that CRH-R1 and CRH-R2 mRNA expression in Fallopian tube from women with ectopic pregnancy is also lower than that observed in the follicular but equivalent to that seen in the luteal phase. We also demonstrate a similar but non-significant trend in CRH-R1 protein expression. Pregnancies with abnormal placental function and vascular resistance are characterized by reduced CRH-R1 expression [31]. CRH treatment, mediated by CRH-R1, causes a reduction in the expression of adhesion molecules and EVT invasion in-vitro [32], and antalarmin, a CRH-R1 specific antagonist, increases EVT invasion in Matrigel assays allowing an uncontrolled trophoblast invasion [14]. Moreover, CRH-R2 is expressed in the uterine vascular bed [20,21] and modulates angiogenesis by inhibiting neovascularization in-vitro [33]. Therefore, we propose that the low CRH-R1 and CRH-R2 mRNA expression observed at the tubal implantation sites may contribute to the chaotic trophoblast invasion and aberrant vascularisation associated with ectopic pregnancy.

We also showed that Ucn1 mRNA expression is lower in decidualized endometrium from women with an ectopic compared to a normal intrauterine pregnancy. A recent study showed that Ucn1 levels are directly related to the degree of decidualization of the endometrium [13] and a direct correlation between Ucn1 concentrations in endometrial flushings and successful implantation has been reported [16]. Additionally, locally produced CRH, which shares 45% homology with Ucn1 [7] and acts with lower affinity than Ucn1 through the same receptors [8,9], has been reported to promote implantation and maintenance of early pregnancy, primarily by inducing apoptosis in activated T cells at the maternal-fetal interface, allowing controlled trophoblast invasion and implantation into maternal decidua [19]. Thus, it is likely that a pregnancy implanted in Fallopian tube has abnormal growth kinetics that are reflected in impaired trophoblast function. Markers of luteal, trophoblast and decidual function are altered in ectopic pregnancy [34-37]. This study confirms an alteration in endometrial secretory function in ectopic pregnancy, possibly due to an absence of trophoblast-derived Ucn1 at the fetal-maternal interface. We are aware that we have only documented statistically significant changes at an mRNA level and that these changes may not necessarily be reflected as alterations in protein expression.

We found that CRH-R1 mRNA expression is considerably higher in the decidualized endometrium of patients with ectopic compared to viable intrauterine pregnancies. CRH-R1 is the only CRH receptor detected in EVT cells and CRH-R1 blockade by antalarmin prevents implantation in rats by reducing the inflammatory-like reaction of the endometrium to the invading blastocyst [19]. The maternal endometrial response to the invading semi-allograft embryo has the characteristics of an acute inflammatory response and, once implanted, the embryo suppresses this response and prevents its rejection. As CRH-R1 has a pro-inflammatory effect in a number of tissues [38,39] and is considered necessary for successful implantation by promoting early embryo-maternal tolerance [19], the increased

decidual CRH-R1 mRNA levels observed in ectopic pregnancy may be due to the absence of intrauterine trophoblast, responsible for the anti-rejection process that protects the fetus from maternal immune system. Alternatively, as it may be assumed that the genetic make-up of the EVT cells is similar in intrauterine and ectopic pregnancies, a pro-inflammatory intrauterine environment leading to abnormal maternal immune tolerance to the fetal semi-allograft and vice versa may contribute to an environment that is not conducive to normal intrauterine implantation and lead to ectopic trophoblast invasion.

This finding was not reflected at the protein level possibly reflecting a degree of variation in the immunohistochemical scoring system and would benefit from quantification by Western blot analysis on isolated epithelial and stromal epithelial cells.

No correlation was observed between Ucn1 mRNA, or CRH-R1 mRNA, and serum progesterone or hCG levels in patients, suggesting that other pathways are involved in the control of Ucn 1/CRH receptor type 1 expression in decidualized endometrium.

In conclusion, this study has demonstrated that: (i) Ucn1 and CRH receptor mRNAs are expressed in the Fallopian tube across the menstrual cycle, complementing data on their expression pattern in the endometrium; (ii) Ucn 1 and CRH receptor mRNA expression is low in the Fallopian tube of women with ectopic pregnancy where tubal implantation has occurred, attributed to deficient tubal decidualization with ectopic pregnancy; (iii) There is a likely imbalance between CRH-R1 and its ligand in the decidualized endometrium of women with ectopic pregnancy, supporting an important role of the CRH family in endometrial differentiation and embryo implantation. However, further work is required to quantify these findings at the protein level.

Acknowledgments

Financial support:

The work was supported by a Wellbeing of Women Project Grant (R40608) to AWH and HODC, and a 2008-2009 Research Grant from the Fondazione Monte dei Paschi di Siena. AWH is supported by an MRC Clinician Scientist Fellowship. The funding source had no role in the study design, data collection, data interpretation, data analysis, or writing of the report.

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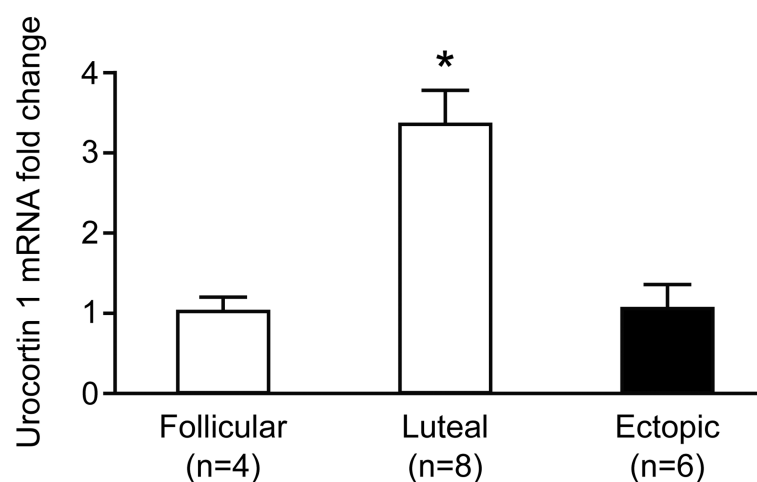


Figure 1. Urocortin 1 mRNA expression in non-pregnant human Fallopian tube across the menstrual cycle and in Fallopian tube from women with ectopic pregnancy

* $P < 0.01$ (luteal versus follicular phase). FP = follicular phase; LP = luteal phase; EP = ectopic pregnancy. Data are mean \pm SEM.

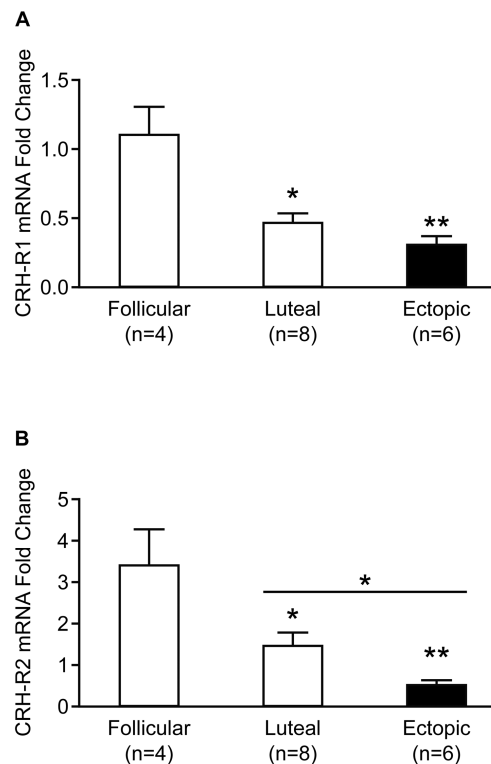


Figure 2. CRH receptor (types 1 and 2) mRNA expression in non-pregnant human Fallopian tube across the menstrual cycle and in Fallopian tube from women with ectopic pregnancy CRH receptor mRNA expression was higher in the follicular phase compared to the luteal phase. CRH receptor mRNA expression was also higher in the follicular phase compared to Fallopian tube from women with ectopic pregnancy. There was no significant difference in CRH₁ receptor expression in the luteal phase compared to Fallopian tube from women with ectopic pregnancy. CRH₂ receptor expression was significantly lower in ectopic pregnancy compared to the luteal phase. * P<0.05 (follicular versus luteal) and **P<0.01 (follicular versus ectopic). EP = ectopic pregnancy. Data are mean \pm SEM.

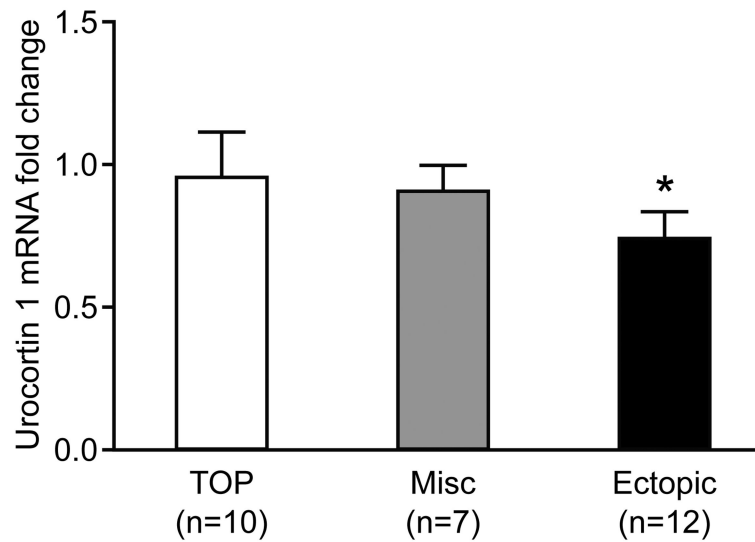


Figure 3. Decidual expression of Urocortin 1 mRNA

Ucn1 expression is higher in decidualized endometrium from viable intrauterine when compared to ectopic pregnancies (** $p < 0.001$). No difference in Ucn1 expression was observed in the decidualized endometrium from non-viable and viable and ectopic pregnancies. TOP = viable intrauterine pregnancy; Misc = non-viable intrauterine pregnancy; EP = ectopic pregnancy. Data are mean \pm SEM.

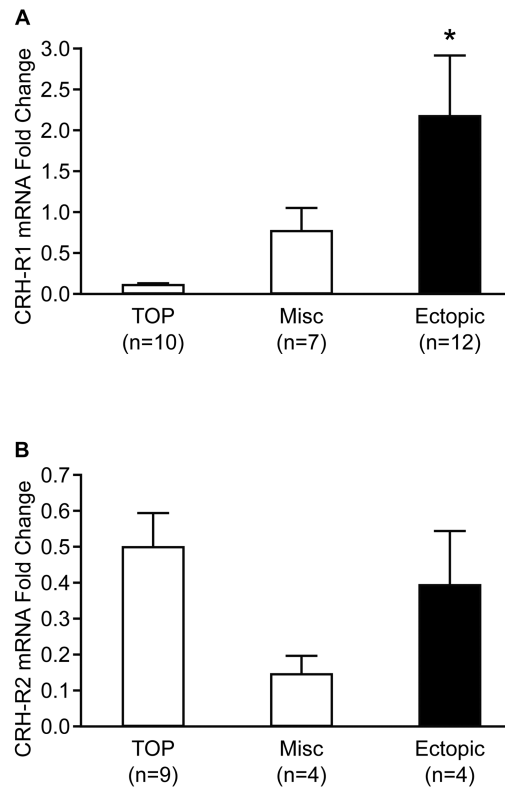


Figure 4. Decidual expression of CRH receptor types 1 and 2 mRNA

CRH receptor type 1 mRNA expression was higher in decidualized endometrium from patients with ectopic compared to viable intrauterine pregnancy (* $p < 0.05$). No difference in CRH receptor type 1 expression was observed between non-viable and ectopic pregnancy. No difference in CRH receptor mRNA expression was observed in the three groups analyzed. TOP = termination of pregnancy (viable intrauterine pregnancy); MISC = miscarriage (non-viable intrauterine pregnancy); Ectopic = ectopic pregnancy. Data are mean \pm SEM.

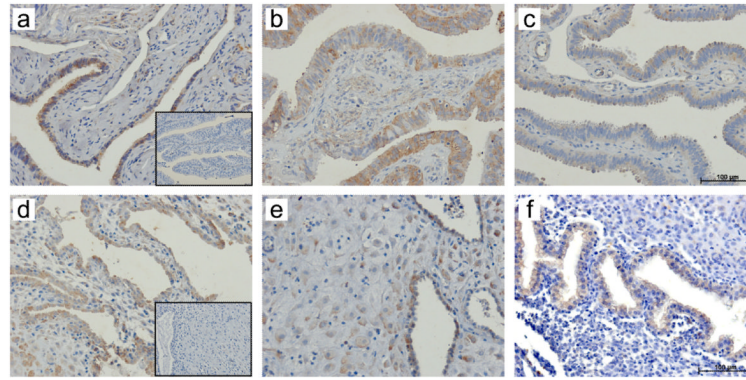


Figure 5. CRH-R1 protein immunolocalisation in Fallopian tube and decidualized endometrium
 CRH-R1 protein was localised to the epithelium of the Fallopian tube. Expression was cytoplasmic with nuclear sparing. There was no difference in localisation at different times of the menstrual cycle or in Fallopian tube from women with ectopic pregnancy. A = Follicular phase; B = Luteal phase; C = Ectopic pregnancy (all Fallopian tube). CRH-R1 showed a similar pattern of expression in the decidualized endometrium although there was evidence of patchy cytoplasmic stromal expression. There was no difference in localisation in the ectopic pregnancy compared to the intrauterine groups. D = Termination of pregnancy (viable intrauterine); E = Miscarriage (non-viable intrauterine); F = Ectopic pregnancy (all decidualized endometrium). Insets are negative controls.

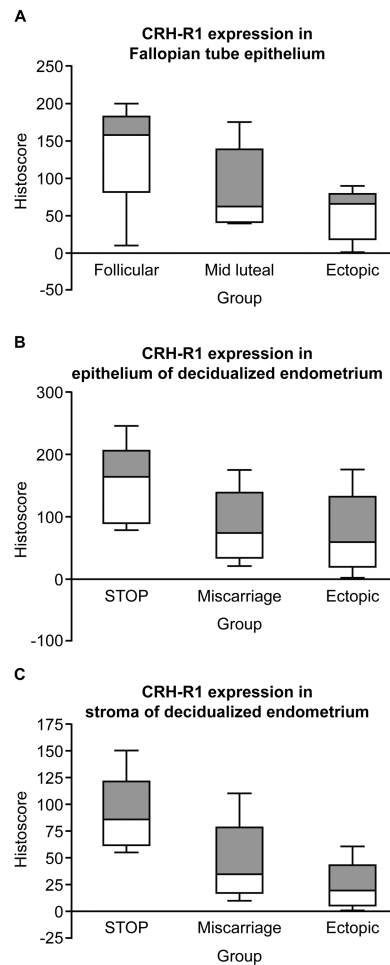


Figure 6. Quantification of CRH-R1 protein expression in Fallopian tube and decidualized endometrium

CRH-R1 protein expression was quantified (n=5) by applying a histoscore (0-300) and presented as box and whisker plots (median and range). None of the differences between each group were statistically significant. However, tubal epithelial CRH-R1 expression showed a non-significant trend reflecting CRH-R1 expression at the mRNA level. TOP = viable intrauterine pregnancy; Misc = non-viable intrauterine pregnancy; EP = ectopic pregnancy.

Table 1

Demographics for Fallopian tube and endometrial biopsies from women undergoing surgery for benign gynaecological conditions.

	Histological dating	Serum estrogen (pmol/l)	Serum progesterone (nmol/l)
1	Follicular	1022.87	0.81
2	Follicular	940.44	3.82
3	Follicular	829.42	4.19
4	Follicular	770.63	5.16
5	Follicular	116.3	2.88
6	Weakly proliferative	55	2.2
7	Mid luteal	242	531
8	Mid luteal	201	24.55
9	Mid luteal	331	83.5
10	Mid luteal	424	76.9
11	Mid luteal	1633	54.38
12	Mid luteal	266	37.1